Disappearance of Erythrocyte Insulin Receptors During Maturation in Sheep

MADHUR K. SINHA, SUPRIYA GANGULI, AND MARK A. SPERLING

SUMMARY

We studied insulin receptor characteristics and glucose uptake in erythrocytes (RBCs) of sheep during maturation. RBC insulin receptors were characterized with respect to binding of monoiodinated ^{125}I-insulin and by Scatchard analysis to determine binding sites, and their affinities. Glucose uptake by RBCs was assessed by incorporation of ^{14}C-2-deoxy D-glucose in the presence of D-glucose. The percent specific ^{125}I-insulin bound to RBCs from fetal sheep (4.81 ± 0.48 SE; N = 11) was significantly higher when compared with those from 1–7-day-old newborn lambs (3.41 ± 0.24, N = 7). Thereafter, insulin binding progressively decreased to 2.45 ± 0.46 at 8–14 days, 0.99 ± 0.08 at 15–21 days, and 0.41 ± 0.25 at 22–35 days. No specific insulin binding was observed in adult sheep RBCs. When individual percent specific ^{125}I-insulin binding was plotted against age, there was a significant negative correlation ($r = -0.79$; $P < 0.01$). Both high (HA) and low (LA) affinity binding sites per erythrocyte were significantly higher in the fetus (HA, 2.2 ± 0.1; LA, 11.6 ± 0.4) compared with those in newborn lambs (HA, 1.3 ± 0.1; LA, 6.4 ± 0.2). Although the association constant (Ka) for HA sites was significantly lower ($P < 0.01$) for fetal RBCs ($2.4 \pm 0.1 \times 10^9$ M^{-1}) compared with that for newborn RBCs ($3.2 \pm 0.1 \times 10^9$ M^{-1}), no significant difference was observed for LA Ka in both groups. Because of low percent specific ^{125}I-insulin binding after 1 wk of age, accurate competition curves could not be calculated. Glucose uptake by fetal sheep RBCs (53 ± 2 nmol/min/L × 10^9 cells, N = 6) was significantly higher than that from adult sheep (25 ± 7, N = 7). A negative correlation existed between RBC glucose uptake and the age of the animals. Although both glucose uptake and insulin receptors decrease postnatally, they appear to be independent phenomena, since insulin (120 μU/ml) did not augment glucose uptake in either fetal cells possessing insulin receptors or adult cells without such receptors. We conclude that disappearance of insulin receptors and decreased glucose uptake in sheep RBCs reflect ongoing and independent maturation of membrane function. **DIABETES *30*:411–415, May 1981.**

From the Division of Endocrinology, Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio 45229.
Address reprint requests to Dr. Sperling.
Received for publication 10 November 1980.

The insulin receptors on erythrocytes,[1,2] like insulin receptors on monocytes,[3] share most of the kinetic characteristics of insulin receptors on target tissues such as adipocytes.[4] Recent studies have described the characteristics of insulin binding to human erythrocytes in both health and disease.[5–8] Studies in various fetal and neonatal tissues suggest that there are substantial differences in the characteristics of the insulin receptors in these tissues when compared with their adult counterparts.[9–11] In both erythrocytes[12–14] and monocytes[15] there is an increase in binding of labeled insulin and an increase in either or both insulin receptor number and affinity in fetal and neonatal cells. In addition, the number of insulin receptors of monocytes[16] and erythrocytes[17] in the fetus does not appear to be influenced by brief exposure to high ambient insulin concentrations and, thus, downregulation is not present. The pre- and postnatal pattern of maturation of insulin receptors on circulating cells has not been completely described for any species. We therefore examined the maturation of insulin receptor characteristics on sheep erythrocytes, in which nutrient sources of energy change dramatically from fetal to adult life. The fetal sheep and newborn lamb are similar to humans and other monogastric species in their requirement and utilization of glucose as a source of energy, while the adult uses volatile fatty acids derived from ruminant fermentation.[18–21] In this report we describe the gradual disappearance of insulin receptors from sheep erythrocytes during postnatal maturation, and the independence of glucose uptake from insulin binding in these cells.

MATERIAL AND METHODS

Reagents. Porcine monocomponent insulin was a gift from Eli Lilly and Company (Indianapolis, Indiana). Na ^{125}I and

deoxy-D-glucose 2-[1-^{14}C] were purchased from New England Nuclear Company and dibutyl phthalate from Aldrich Chemicals. Other chemicals were of analytic grade from Sigma Chemical Company and Eastman Kodak Company.

Blood Samples. Blood samples were obtained from chronically catheterized fetal sheep of known gestational age, from the same animals at intervals after delivery, and from nonpregnant female and male adult sheep. To validate the methods and to compare the results with published data, blood was also obtained from seven normal healthy male volunteers, after an overnight fast; they had no family history of diabetes mellitus or other disorders and were within 10% of ideal body weight.

Isolation of Erythrocytes. The erythrocytes were isolated from other cells by a modified Boyum's method as described by Gambhir et al.[1] In brief, after centrifugation of heparinized blood the plasma was aspirated, the cell pellet diluted with one part of Dulbecco's phosphate-buffered saline (PBS), layered on Ficoll-Hypaque gradient (lymphocyte separation medium, Bionetics), and centrifuged at 400 × *g* for 30 min at room temperature. The buffer, lymphocytes, gradient, and top of the erythrocyte layer were removed. The whole procedure was repeated once, and the resulting pellet was washed twice with assay buffer and suspended in assay buffer, to yield erythrocyte concentration of 4–5 × 10^9 cells/ml. Leukocytes and platelets were only rarely seen on Wright's stained smears of such erythrocyte preparations. Reticulocytes were present in ¼ to ½ of their original concentration in the collected blood samples. Nucleated erythrocytes were occasionally seen in the preparations from fetal sheep blood, however none of the binding characteristics of the erythrocyte preparations correlated with the presence of these other cells. The viability of erythrocytes was always more than 95%, as measured by trypan blue exclusion test. For glucose uptake studies the erythrocytes were suspended in 1% BSA-PBS, in a final concentration of 4–5 × 10^9 cells/ml.

Insulin binding to erythrocyte receptors. Monoiodinated ^{125}I-insulin was prepared by the method of Sodoyez et al.[22] The specific activity of various batches of monoidinated ^{125}I-insulin ranged between 100–150 μCi/μg.

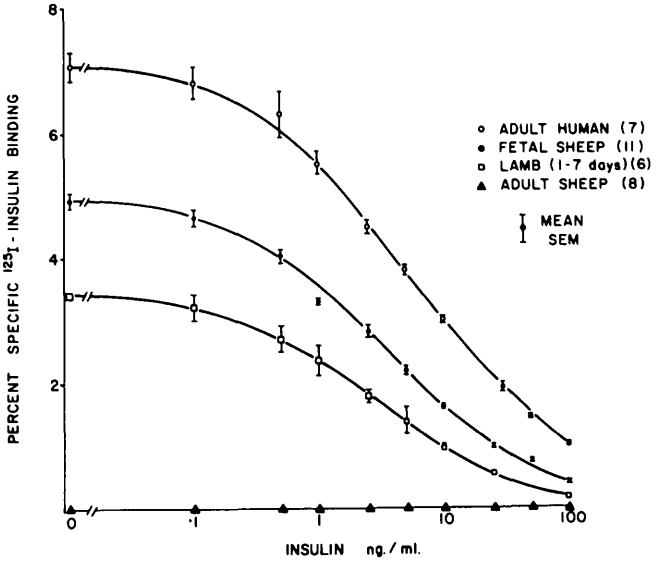
The method for insulin binding to erythrocyte receptor was as reported by Gambhir et al.[1] Aliquots (0.4 ml) of original erythrocyte suspension containing 1.6–2.2 × 10^9 cells were incubated in assay buffer (Hepes 50 mM, Tris 50 mM, $MgCl_2$ 10 mM, EDTA 2 mM, dextrose 10 mM, $CaCl_2$ 10 mM, NaCl 50 mM, KCl 5 mM, and 0.1% BSA, pH 8.0) with ^{125}I-insulin (80 pg), in the presence or absence of unlabeled insulin, 0.1–100 ng/ml, for 180 min at 15°C in a final volume of 0.5 ml. Thus, the final cell concentration in the incubation medium was 3.2–4.4 × 10^9 cells/ml. After incubation, 0.2-ml aliquots in duplicate were transferred to prechilled microfuge tubes containing 0.2 ml assay buffer and 0.2 ml dibutyl phthalate and cetrifuged in a Beckman microfuge for 2.5 min. The supernatant was aspirated, the cell pellet was excised, and radioactivity counted in an autogamma counter. Nonspecific binding, measured as residual radioactivity in the presence of excess of unlabeled insulin (100 μg/ml), was subtracted appropriately from total binding. For comparison between different groups all insulin binding data were normalized for 4 × 10^9 cells/ml. Binding curves were analyzed by the graphical method of Scatchard.[23] Degradation of ^{125}I-insulin during incubation with erythrocytes was measured by precipitating the supernatant with 5% trichloroacetic acid.

Glucose uptake by erythrocytes. The method for measuring glucose uptake is based on the similarity in kinetics of radiolabeled 2-deoxyglucose and D-glucose transport across the cell membrane, and takes advantage of the fact that 2-deoxyglucose cannot be metabolized after phosphorylation.[24] Erythrocytes (0.8–1.0 × 10^9 cells/ml) were incubated in physiologic phosphate-buffered saline, pH 7.4, with 1% BSA and with 0.7 μM [1-^{14}C] 2-deoxyglucose and 10 μM glucose for 10 min at 24°C; the total volume was 0.5 ml. After the addition of 2 ml of chilled phosphate buffer, the tubes were centrifuged at 1000 × *g* for 10 min and the supernatant aspirated. Erythrocytes were then lysed by adding 0.8 ml water and the hemoglobin precipitated by trichloroacetic acid. After a further centrifugation, the clear supernatant was transferred to vials containing 10 ml aqueous counting scintillant (Amersham) and ^{14}C-radioactivity was counted in a Mark III Searle liquid scintillation counter. The radioactivity in the erythrocytes at zero time at 4°C represented the amount of glucose left in the extracellular aqueous phase, which was less than 1% of the total radioactivity. All data for glucose uptake were corrected for extracellular trapping. Glucose uptake was derived as the incorporation of radioactive 2-deoxyglucose/min/10^9 cells. In preliminary experiments, the glucose uptake was linear up to 30 min and directly proportional to the cell concentration. The V_{max} in this system was reached at a glucose concentration of 10 μM. Statistical analysis was performed by Student's *t* test and by least square regression, using a programmable calculator.

RESULTS

Figure 1 shows the competition curves of ^{125}I-insulin binding to erythrocytes from adult humans and from fetal (122–140 days; term ≃ 145 days), postnatal (1–7 days), and adult sheep. The percent ^{125}I-insulin bound to erythrocytes from fetal sheep was significantly higher when compared

FIGURE 1. Dose-response curves: the percent specific ^{125}I-insulin binding to erythrocytes (4 × 10^9 cells/ml) is plotted against total insulin concentration (ng/ml).



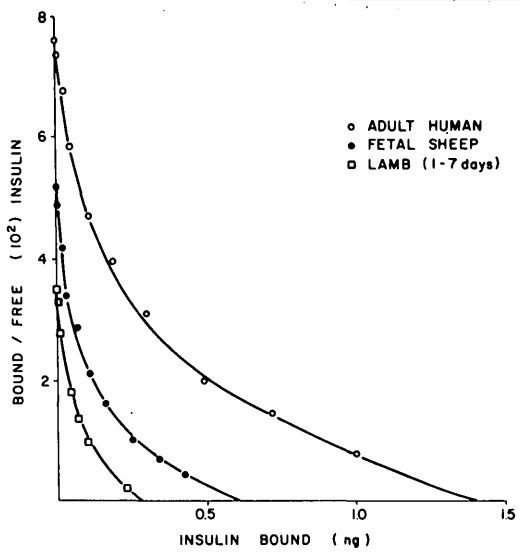


FIGURE 2. Scatchard plots of the dose-response curves from Figure 1.

with that in the 1–7-day-old lambs ($P < 0.01$). There was no specific ^{125}I-insulin binding to erythrocytes from adult sheep. In fetal sheep, the insulin binding was significantly less ($P < 0.05$) when compared with adult humans, for whom the binding characteristics presented here are similar to results reported in other studies.[13–14] Scatchard analyses of these binding data are presented in Figure 2, and receptor characteristics are presented in Table 1. Total receptor number was significantly less in fetal sheep than in adult humans ($P < 0.01$) and was still lower in newborn lambs ($P < 0.01$). Resolution of these curvilinear Scatchard plots into their high (HA) and low (LA) affinity components by the method of Rosenthal[25] revealed that the affinity of both components was significantly higher in fetal and newborn lambs when compared with adult humans ($P < 0.01$). The high affinity component in the lamb was significantly greater than that of the fetus ($P < 0.01$), but no significant differences existed for the low affinity component ($P < 0.05$). The average affinity constant ($\bar{K}$) was calculated and plotted against fractional occupancy ($\bar{y}$) to provide and average affinty profile.[26] The average affinity of insulin binding sites in the empty configuration ($\bar{K}e$), and in the filled configuration ($\bar{K}f$) are shown in Table 1.

The changes in insulin binding, receptor sites per cell, percent reticulocytes, and glucose during maturation in sheep are illustrated in Figure 3. There is a progressive decrease in specific binding during both pre- and postnatal periods. The percent specific ^{125}I-insulin binding gradually decreases ten-fold from 4.81 ± 0.48 in fetal sheep to 0.41 ± 0.25 in 4–5-wk-old lambs. After this age we could not demonstrate any specific ^{125}I-insulin binding. In addition, because of the low specific binding after 1 wk of age, accurate competition curves could not be calculated, precluding the determination of receptor number and affinity. The lack of any specific ^{125}I-insulin binding in adult sheep erythrocytes was not due to excessive degradation of the label; percent degradation at the end of 3-h incubation was 17.4 ± 0.9 in adult sheep, compared with 13.9 ± 0.6 in adult humans. In addition, changes in insulin binding occurred independently of changes in the reticulocyte counts during the initial 2 wk of postnatal life. Furthermore, adult human RBCs with low reticulocyte count bound considerably more insulin than fetal or newborn lamb RBCs.

The glucose uptake by erythrocytes of fetal sheep was similar to that of adult humans, but was significantly higher than that from adult sheep ($P < 0.01$). Insulin (120 μU/ml) did not alter glucose uptake (nmol/min/10^9 cells) by erythrocytes either from fetal or adult sheep (fetal 48.3 ± 8.5 versus 52.9 ± 13.9, N = 4; adult 25.2 ± 4.6 versus 23.8 ± 3.7, N = 4). When individual values of specific ^{125}I-insulin binding to erythrocytes were plotted against age, there was significant negative correlation ($r = -0.79$, $P < 0.01$). Similarly, there was a negative correlation between glucose uptake and the age of the animals ($r = -0.50$, $P < 0.05$, Figure 4).

DISCUSSION

Erythocytes are an easily accessible cell for the assessment of insulin receptor characteristics. Several studies in humans describe increased receptor number in pre-term and full-term infants compared with adults.[13–14] To validate our binding data for sheep, we measured insulin binding to adult human erythrocytes and found the percent specific insulin binding and the total number of insulin receptors per cell to be comparable to results reported in other recent studies.[13,14] Also, the association constants for low and high affinity sites were comparable with those reported for monocytes,[27] hepatocytes,[28] and adipocytes.[29]

Because fetal growth and development is promoted by the presence of insulin, as observed in the fetal overgrowth of hyperinsulinemic states and growth retardation of insulinopenic states,[30] the increased receptor number in the fetus and newborn may reflect the anabolic influence of insulin in utero. As reported in fetal and newborn humans, we also found a negative correlation between insulin receptor num-

TABLE 1
Comparison of the affinity and number of insulin receptors in fetal sheep, newborn lambs, and adult humans

	Ka		Sites/cell			$\bar{K} \times 10^8$ M^{-1}	
	HA (× 10^9 M^{-1})	LA (× 10^8 M^{-1})	Total	HA	LA	$\bar{K}e$	$\bar{K}f$
Fetal sheep (11)	2.4 ± 0.1*	2.2 ± 0.1*	13.5 ± 0.4*	2.2 ± 0.1*	11.7 ± 0.4*	14.4	4.1
Newborn lambs (7) (1–7 days)	3.2 ± 0.1*†	2.2 ± 0.2*	7.8 ± 0.2*†	1.3 ± 0.1*†	6.4 ± 0.2*†	20.5	8.5
Adult humans (7)	1.8 ± 0.2	1.2 ± 0.2	49.1 ± 1.4	4.8 ± 0.3	44.4 ± 3.6	9.3	2.7

* Adult human versus fetal sheep or newborn lambs, $P < 0.01$.
† Fetal sheep versus newborn lambs, $P < 0.01$.
Data are presented as mean ± SEM.
See text for definition of Ka, HA, LA, $\bar{K}$, $\bar{K}e$, and $\bar{K}f$.

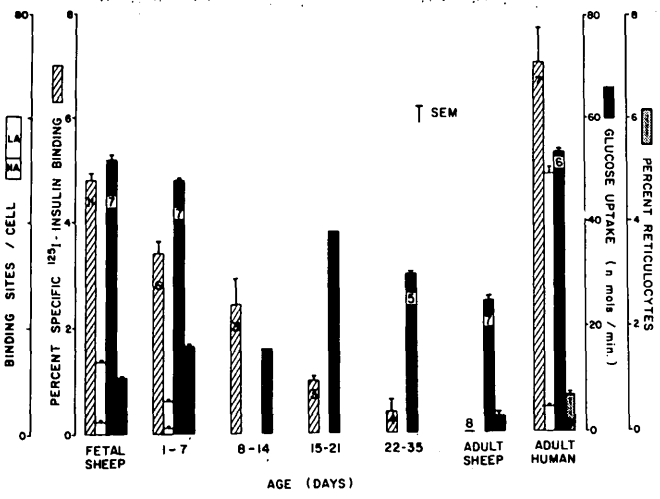
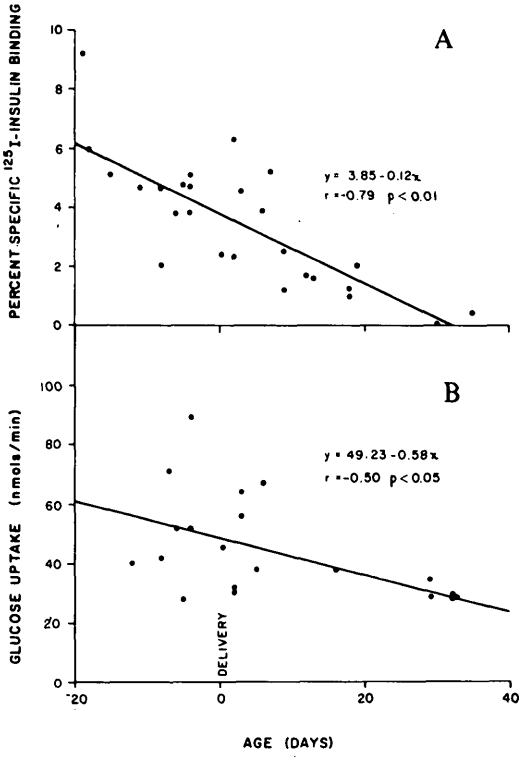


FIGURE 3. Percent specific ^{125}I-insulin binding, number of high (HA) and low (LA) affinity binding sites per cell, glucose uptake by erythrocytes (nmol/min/10^{9}cells), and percent reticulocyte count in the blood from fetal sheep, lambs of different postnatal ages, adult sheep, and adult humans.

ber and gestational or postnatal age in fetal and newborn sheep. However, unlike humans, in which insulin receptors on erythrocytes persist (although reduced in number), our observations show that the fetal population of insulin receptors disappears in adult sheep. The factors contributing to this loss of insulin receptors in sheep are not clear. Although studies in rabbit and humans indicate that there is considerably greater insulin binding in younger cells,[31,32] we show that the progressive loss of insulin receptors in maturing sheep cannot be related to changes in the proportion of reticulocytes; despite comparatively low reticulocyte counts in human blood, insulin binding and insulin receptor number were significantly higher than in the blood of fetal or newborn lambs. Finally, the loss of insulin receptors in adult sheep erythrocytes could not be explained by greater degradation of labeled hormone. Downregulation of insulin receptor number also cannot explain the disappearance of insulin binding in adult sheep. The circulating insulin concentrations in mature sheep are not significantly different than those in fetal sheep,[33] in spite of the metabolic adaptation from glucose to volatile fatty acids as a major source of energy during maturation.[18-21] In this regard, it is striking that glucose uptake by erythrocytes also progressively decreases. Using methods that differed from those in the present study, complete disappearance of glucose uptake by erythrocytes of adult sheep[34,35] and guinea pigs[36] has been reported. Progressive disappearance of specific glucose transport proteins from the cell membrane of guinea pig erythrocytes has also been demonstrated.[36] Thus, the progressive decrease in glucose transport in maturing sheep erythrocytes suggests a similar alteration in the membrane transport system for glucose, and the loss of insulin receptors during maturation represents a further example of ongoing membrane differentiation. Although temporally related, erythrocyte glucose uptake and insulin receptors in sheep are not causally related, since glucose transport is independent of insulin concentration—even in fetal cells possessing insulin receptors.

FIGURE 4. Correlation between percent specific ^{125}I-insulin binding (A) or glucose uptake (B) by erythrocytes and age of pre- and postnatal sheep.



ACKNOWLEDGMENTS

This work was supported, in part, by grant no. HD 12613 from the USPHS.

REFERENCES

[1] Gambhir, K. K., Archer, J. A., and Bradley, C. J.: Characteristics of human erythrocyte insulin receptors. Diabetes *27*:701–08, 1978.

[2] Insel, J. R., Kolterman, O. G., Saekow, M., and Olefsky, J. M.: Short-term regulation of insulin receptor affinity in man. Diabetes *29*:132–39, 1980.

[3] Gavin, J. R., III, Roth, J., Jen, P., and Freychet, P.: Insulin receptors in human circulating cells and fibroblasts. Proc. Natl. Acad. Sci. USA *69*:747–51, 1972.

[4] Olefsky, J. M.: Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J. Clin. Invest. *57*:1165–72, 1976.

[5] Robinson, T. J., Archer, J. A., Gambhir, K. K., Hollis, V. W., Jr., Carter, L., and Bradley, C.: Erythrocytes: a new cell for the evaluation of insulin receptor defects in diabetic humans. Science *205*:200–02, 1979.

[6] Wachslicht-Rodbard, H., Gross, H. A., Rodbard, D., Ebert, M. H., and Roth, J.: Increased insulin binding to erythrocytes in anorexia nervosa. N. Engl. J. Med. *300*:882–87, 1979.

[7] Pedersen, O., Beck-Nielsen, H., and Heding, L.: Increased insulin receptors after exercise in patients with insulin-dependent diabetes mellitus. N. Engl. J. Med. *302*:886–92, 1980.

[8] DePirro, R., Fusco, A., Lauro, R., Testa, I., Ferreti, F., and DeMartinis, C.: Erythrocyte insulin receptors in non-insulin-dependent diabetes mellitus. Diabetes *29*:96–99, 1980.

[9] Kelley, P. A., Posner, B. I., Tsushima, T., and Friesen, H. G.: Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. Endocrinology *95*:532–39. 1974.

[10] Ganguli, S., Voina, S., Velayo, N., Devaskar, U., and Sperling, M. A.: Ontogenesis of insulin and glucagon receptors and adenylate cyclase in rabbit fetal liver plasma membrane. Diabetes *29* (Suppl. 2):102A, 1980. Abstract.

[11] Vinicor, F., and Clark, C., Jr.: Characterization and significance of hepatic receptors for insulin during fetal and neonatal life. Diabetes *29*(Suppl. 2):38A, 1980. Abstract.

[12] Hill, D. E., Victoria, L., Herzberg, J., Boughter, J. M., Carlisle, S. K., and Sziszak, T. J.: Insulin binding in fetal and maternal guinea pig erythrocytes. Pediatr. Res. *13*:359, 1980. Abstract.

[13] Kappy, M. S., and Plotnick, L.: Studies of insulin binding in children using human erythrocytes in small amounts of blood. Diabetes *28*:1001–05, 1979.

[14] Herzberg, V. L., Boughter, J. M., Carlisle, S. K., Ahmad, F., and Hill, D. E.: ^{125}I-insulin receptor binding to cord blood erythrocytes of varying gestational age and comparison with adult values. Pediatr. Res. *14*:4–7, 1980.

[15] Thorsson, A. V., and Hintz, R. L.: Insulin receptors in newborn: increase in receptor affinity and number. N. Engl. J. Med. *297*:908–12, 1977.

[16] Neufeld, N. D., Kaplan, S. A., Lippe, B. M., and Scott, M.: Increased monocyte receptor binding of ^{125}I-insulin in infants of diabetic mothers. J. Clin. Endocrinol. Metab. *47*:590–95, 1978.

[17] Kaufman, E., Allen, R., Eaton, R. P., and Perkins, R.: Cord blood erythrocyte binding of ^{125}I-insulin: the effect of fetal hyperinsulinemia. Pediatr. Res. *14*:575, 1980. Abstract.

[18] McCandless, E. L., and Dye, J. A.: Physiological changes in intermediatry metabolism of various species of ruminants incident of functional development of rumen. Am. J. Physiol. *162*:434–46, 1950.

[19] Jarret, I. G., Jones, G. B., and Potter, B. J.: Changes in glucose utilization during development of lamb. Biochem. J. *90*:189–94, 1964.

[20] Ballard, F. J., and Oliver, I. T.: Carbohydrate metabolism in liver from fetal and neonatal sheep. Biochem. J. *95*:191–200, 1965.

[21] Battaglia, F. C., and Meschia, G.: Principal substrates of fetal metabolism. Physiol. Rev. *58*:499–527, 1978.

[22] Sodoyez, J. C., Goffaux, F. S., Goff, M. M., Zimmerman, A. G., and Arquilla, E. R.: [^{127}I]- or carrier-free [^{125}I] monoiodoinsulin. Preparation, physical, immunological and biological properties and susceptibility to "insulinase" degradation. J. Biol. Chem. *250*:4268–77, 1975.

[23] Scatchard, G.: The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci. *51*:660–72, 1949.

[24] Wick, A. N., Drury, D. R., Nakada, H. I., and Wolfe, J. B.: Localization of primary metabolic block produced by 2-deoxy glucose. J. Biol. Chem. *224*:963–69, 1957.

[25] Rosenthal, H. E.: A graphic method for the determination and presentation of binding parameters in a complex system. Anal. Biochem. *20*:525–32, 1967.

[26] DeMeyts, P., and Roth, J.: Cooperativity in ligand binding: a new graphic analysis. Biochem. Biophys. Res. Commun. *66*:1118–26, 1975.

[27] Schwartz, R. H., Blanco, A. R., Handwerger, B. S., and Kahn, C. R.: Demonstration that monocytes rather than lymphocytes are the insulin binding cells in preparation of human peripheral blood mononuclear leukocytes preparation: implication for studies of insulin resistance states in man. Proc. Natl. Acad. Sci. USA *72*:474–78, 1975.

[28] Cuatrecasas, P., Desbuquois, B., and Krug, F.: Insulin receptor interactions in liver cell membranes. Biochem. Biophys. Res. Commun. *44*:333–39, 1971.

[29] Olefsky, J. M., Jen, P., and Reaven, G. M.: Insulin binding to isolated human adipocytes. Diabetes *23*:565–71, 1974.

[30] Hill, D.: Diabetes and Other Endocrine Disorders During Pregnancy and in the Newborn. New York, Alan R. Liss, 1976, p. 127.

[31] Thomopoulos, P., Berthellier, M., and Laudt, M.: Loss of insulin receptors on maturation of reticulocytes. Biochem. Biophys. Res. Commun. *85*:1460–65, 1978.

[32] Eng, J., Lilly, L., and Yalow, R. S.: Influence of age of erythocytes on their insulin receptors. Diabetes *29*:164–66, 1980.

[33] Anand, R. S., Ganguli, S., and Sperling, M. A.: Effect of insulin induced maternal hypoglycemia on fetal-maternal glucose production, utilization and placental transfer in sheep. Am. J. Physiol. *238*:E524–32, 1980.

[34] Widdas, W. F.: Hexose permeability of fetal erythrocytes. J. Physiol. *127*:318–27, 1955.

[35] Laris, P. C.: Permeability and utilization of glucose in mammalian erythrocytes. J. Cell. Physiol. *51*:273–307, 1958.

[36] Kondo, T., and Beutler, E.: Developmental changes in glucose transport of guinea pig erythrocytes. J. Clin. Invest. *65*:1–4, 1980.